

Amendments to the Specification

Please amend the specification as follows:

At page 1, please replace the paragraph at lines 6-18 with:

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-- Related Applications

C<sub>1</sub>  
This application is a continuation application of U.S. Ser. No. 08/403,253, filed March 10, 1995, entitled "Methods for Selectively Stimulating Proliferation of T Cells" (now U.S. Pat. No. 6,352,694); which in turn is a continuation-in-part of U.S. Ser. No. 08/253,964, filed June 3, 1994, entitled "Methods for Selectively Stimulating Proliferation of T Cells" (currently pending). The contents of the aforementioned applications are hereby incorporated by reference. --

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At page 24, please replace the paragraph at lines 22-38 continuing to page 25, lines 1-2 with:

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C<sub>2</sub>  
-- To maintain long term stimulation of a population of T cells following the initial activation and stimulation, it is necessary to separate the T cells from the activating stimulus (e.g., the anti-CD3 antibody) after a period of exposure. The T cells are maintained in contact with the co-stimulatory ligand throughout the culture term. The rate of T cell proliferation is monitored periodically (e.g., daily) by, for example, examining the size or measuring the volume of the T cells, such as with a ~~Coulter~~ Coulter COUNTER COUNTER®. A resting T cell has a mean diameter of about 6.8 microns. Following the initial activation and stimulation and in the presence of the stimulating ligand, the T cell mean diameter will increase to over 12 microns by day 4 and begin to decrease by about day 6. When the mean T cell diameter decreases to approximately 8 microns, the T cells are reactivated and restimulated to induce further proliferation of the T cells. Alternatively, the rate of T cell proliferation and time for T cell restimulation can be monitored by assaying for the presence of cell surface

C<sub>2</sub> molecules, such as B7-1, B7-2, which are induced on activated T cells. As described in Example 5, it was determined that CD4<sup>+</sup> T cells do not initially express the B7-1 receptor, and that with culture, expression is induced. Further, the B7-1 expression was found to be transient, and could be re-induced with repeated anti-CD3 restimulation. Accordingly, cyclic changes in B7-1 expression can be used as a means of monitoring T cell proliferation; where decreases in the level of B7-1 expression, relative to the level of expression following an initial or previous stimulation or the level of expression in an unstimulated cell, indicates the time for restimulation. - -

At page 38, please replace the paragraph at lines 18-38 with:

C<sub>3</sub> - - The cell sorting via negative magnetic immunoadherence must be performed at 4°C. The washed cell pellets obtained from the PERCOLL<sup>TM</sup> gradients described above were resuspended in coating medium (RPMI-1640 (Bio Whittaker, Walkersville, Md., Catalog # 12-167Y), 3% fetal calf serum (FCS) (or 1% human AB<sup>-</sup> serum or 0.5% bovine serum albumin) 5 mM EDTA (Quality Biological, Inc., Gaithersburg, Md., Catalog # 14-117-1), 2 mM L-glutamine (Bio Whittaker, Walkersville, Md., Catalog # 17-905C), 20 mM HEPES (Bio Whittaker, Walkersville, Md., Catalog # 17-757A), 50 µg/ml gentamicin (BioWhittaker, Walkersville, Md., Catalog # 17-905C)) to a cell density of 20 X 10<sup>6</sup> per ml. A cocktail of monoclonal antibodies directed to cell surface markers was added to a final concentration of 1 µg/ml for each antibody. The composition of this cocktail is designed to enrich for either CD4<sup>+</sup> or CD28<sup>+</sup> T cells. Thus, the cocktail will typically include antibodies to CD14, CD20, CD11b, CD16, HLA-DR, and (for CD4<sup>+</sup> cells only) CD8. (See Table 1 for a list of sorting monoclonal antibody cocktails.) The tube containing cells and antibodies was rotated at 4°C for 30-45 minutes. At the end of this incubation, the cells were washed three times with coating medium to remove unbound antibody. Magnetic beads coated with goat anti-mouse IgG (~~Dynabeads M-450~~

C<sub>3</sub> DYNABEADS® M-450, Catalog #11006, P&S Biochemicals, Gaithersburg, Md.) and prewashed with coating medium were added at a ratio of three beads per cell. The cells and beads were then rotated for 1-1.5 hours at 4°C. The antibody-coated cells were removed using a magnetic particle concentrator according to the manufacturer's directions (MPC-1, Catalog # 12001, P&S Biochemicals, Gaithersburg, Md.). The nonadherent cells were washed out of the coating medium and resuspended in an appropriate culture medium. - -

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At page 40 please replace the paragraph at lines 4-19 with:

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C<sub>4</sub> - - Tissue culture flasks precoated with anti-CD3 monoclonal antibody were thawed and washed three times with PBS. The purified T cells were added at a density of  $2 \times 10^6$ /ml. Anti-CD28 monoclonal antibody mAb 9.3 (Dr. Jeffery Ledbetter, Bristol Myers Squibb Corporation, Seattle, Wash.) or EX5.3D10, ATCC Deposit No. HB11373 (Repligen Corporation, Cambridge, Mass.) was added at a concentration of 1 µg/ml and cells were cultured at 37 °C overnight. The cells were then detached from the flask by forceful pipetting and transferred to a fresh untreated flask at a density of  $0.5 \times 10^6$ /ml. Thereafter, the cells were resuspended every other day by forceful pipetting and diluted to  $0.5 \times 10^6$ /ml. The mean diameter of the cells was monitored daily with a ~~Coulter Counter 2M~~ COULTER COUNTER® 2M interfaced to a Coulter Channelyzer. Resting T cells have a mean diameter of 6.8 microns. With this stimulation protocol, the mean diameter increased to over 12 microns by day 4 and then began to decrease by about day 6. When the mean diameter decreased to about 8 microns, the cells were again stimulated overnight with anti-CD3 and anti-CD28 as above. It was important that the cells not be allowed to return to resting diameter. This cycle was repeated for as long as three months. It can be expected that the time between restimulations will progressively decrease. - -

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At page 44, please replace the paragraph at lines 17-26 with:

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-- To determine the time of T cell restimulation, changes in cell volume were monitored using a ~~Coulter Counter ZM~~ COULTER COUNTER® ZM interfaced with a Coulter. CD28<sup>+</sup> CD4<sup>+</sup> T cells were isolated as described by magnetic immunoselection, and cultured in the presence of anti-CD28 mAb 9.3 (0.5 µg /ml) and restimulated with plastic immobilized anti-CD3 monoclonal antibody G19-4 as indicted. FIG. 9 demonstrates the cyclic changes in cell volume during six consecutive restimulations ("S1" to "S6") performed essentially as described in Example 1. Briefly, cells were expanded with anti-CD3 and anti-CD28 over three weeks in culture. Cells were changed to fresh medium at each restimulation with anti-CD3 antibody. Stimulations were spaced at ten day intervals. The cells were restimulated whenever cell volume decreased to <400 fl. --

At page 47, please replace the paragraph at lines 18-31 with:

-- **Example 9: Long Term Growth of CD8<sup>+</sup> T cells With Anti-CD3 and Monoclonal Antibody 2D8 ES5.2D8**

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Experiments were conducted to determine whether a population of CD8<sup>+</sup> T cells could be preferentially expanded by stimulation with an anti-CD3 mAb and a monoclonal antibody ~~2D8~~ ES5.2D8. CD28<sup>+</sup> T cells were obtained essentially as described in Example 1. To assay for CD8 expression, a primary anti-CD8 antibody and a labeled appropriate secondary antibody were used in FACS analysis to determine the percent positive cells. As shown in FIG. 17, at day 7 following stimulation of T cells with the anti-CD3 mAb G19-4sp and the mAb ~~2d8~~ ES5.2D8, the CD8<sup>+</sup> fraction had increased from approximately 20% to over 40%. Another monoclonal antibody ER4.7G11 (referred to as 7G11) was also found to stimulate CD8<sup>+</sup> T cells. This antibody was raised against recombinant human CTLA4 and has been deposited with the ATCC

C<sub>6</sub> on Jun. 3, 1994 at Accession No. HB 11642. This result indicates that binding of either a distinct region of CTLA4 or of a cross-reactive cell surface protein selectively activates CD8<sup>+</sup> T cells. - -

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At page 47, please replace the paragraph beginning at lines 33-38 and continuing on page 48, lines 1-9 with:

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- - Example 10: Defining the Epitope of the Monoclonal Antibody 2D8 ES5.2D8 and Cloning the CD9 Antigen

C<sub>7</sub> To ~~determine~~ determine the epitope of the monoclonal antibody ~~2D8~~ ES5.2D8, epitope mapping was performed by phage display library (PDL) screening and was confirmed using synthetic peptides. A random 20 amino acid PDL was prepared by cloning a degenerate oligonucleotide into the fUSE5 vector (Scott, J. K. and Smith, G. P. (1990) Science 249:386-390) as described in Cwirla, S. E. et al.. (1990) Proc. Natl. Acad. Sci. USA 87:6378-6382. The PDL was used to identify short peptides that specifically bound mAb ~~2D8~~ ES5.2D8 by a micropanning technique described in Jellis, C. L. et al.. (1993) Gene 137:63-68. Individual phage clones were purified from the library by virtue of their affinity for immobilized mAb and the random peptide was identified by DNA sequencing. Briefly, mAb ~~2D8~~ ES5.2D8 was coated onto Nunc Maxisorp 96 well plates and incubated with  $5 \times 10^{10}$  phage representing  $8 \times 10^6$  different phage displaying random 20 amino acid peptides. Specifically bound phage were eluted, amplified, then incubated with the antibody a second time. After the third round, 7 phage were isolated, and DNA was prepared for sequencing. - -

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At page 48, please replace the paragraph at lines 20-21 with:

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C<sub>8</sub> - - In addition to CTLA4, a second antigen for mAb ~~2D8~~ ES5.2D8 was discovered using cDNA expression cloning. - -

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At page 50, please replace the paragraph at lines 9-16 with:

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-- B. Cloning Procedure

In the cloning procedure, the cDNA expression library was introduced into MOP8 cells (ATCC No. CRL1709) using lipofectamine and the cells screened with mAb ~~2D8~~ ES5.2D8 to identify transfectants expressing a ~~2D8~~ ES5.2D8 ligand on their surface.

C<sub>9</sub> In the first round of screening, thirty 100 mm dishes of 50% confluent COS cells were transfected with 0.05 µg /ml activated T cell library DNA using the DEAE-Dextran method (Seed, B. et al.. (1987) Proc. Natl. Acad. Sci. USA 84:3365). The cells were trypsinized and re-plated after 24 hours. After 47 hours, the cells were detached by incubation in PBS/0.5 mM EDTA, pH 7.4/0.02% Na azide at 37 °C. for 30 min. --

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At page 50, please replace the paragraph at lines 17-23 with:

-- Detached cells were treated with 10 µg/ml mAb ~~2D8~~ ES5.2D8. Cells were incubated with the monoclonal antibody for 45 minutes at 4 °C. Cells were washed and distributed into panning dishes coated with affinity-purified goat anti-mouse IgG antibody and allowed to attach at room temperature. After 3 hours, the plates were gently washed twice with PBS/0.5 mM EDTA, pH 7.4/0.02% Na azide, 5% FCS and once with 0.15M NaCl, 0.01M Hepes, pH 7.4, 5% FCS. Unbound cells were thus removed and episomal DNA was recovered from the adherent panned cells by conventional techniques. --

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At page 50, please replace the paragraph at lines 24-30 with:

C<sub>11</sub> -- Episomal DNA was transformed into E. coli DH10B/P3. The plasmid DNA was re-introduced into MOP8 cells using lipofectamine and the cycle of expression and panning was repeated twice. Cells expressing a ~~2D8~~ ES5.2D8 ligand were selected by panning on dishes coated with goat anti-mouse IgG antibody. After the third round of

C 11  
screening, plasmid DNA was prepared from individual colonies and transfected into MOP8 cells by the DEAE-Dextran method. Expression of a ~~2D8~~ ES5.2D8 ligand on transfected MOP8 cells was analyzed by indirect immunofluorescence with mAb ~~2D8~~ ES5.2D8 (See FIG. 18). --

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At pages 50, please replace the paragraph beginning at lines 3-9 and continuing on page 51, lines 1-2 with:

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-- BESTFIT analysis of the phage epitopes of mAb ~~2D8~~ ES5.2D8 to the amino acid sequence of CD9 revealed a close match:

C 12  
G C W L L R E (phage 2D8#2, 4, 10; SEQ ID NO: 11)

G I W L R P D (phage 2D8#6; SEQ ID NO: 12)

G L W L R F D (CD9 sequence; SEQ ID NO: 13) --

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At page 54, please replace the paragraph beginning at lines 28-38 and continuing on page 55, lines 1-10 with:

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C 13  
-- The ability of the B7 ligands to sustain T cell proliferation in long term cultures was investigated. For these experiments, CD4<sup>+</sup> T cells were obtained from CD28<sup>+</sup> T cells by negative selection using magnetic beads (Dynal) coated with CD8 monoclonal antibodies as described in June et al.. (1989) J. Immunol. 143:153. The phenotype of the cells was 99% CD2<sup>+</sup> 98% CD28<sup>+</sup>, and 96% CD4<sup>+</sup>. 5 X 10<sup>6</sup> purified CD4<sup>+</sup> T cells were stimulated with anti-CD3 monoclonal antibody coated beads (1.5 X 10<sup>7</sup> beads) and mitomycin C-inactivated CHO cells (2 X 10<sup>6</sup> cells) expressing B7-1, B7-2, or neomycin resistance only, or with anti-CD3 plus anti-CD28 coated "cis" beads, i.e. with both antibodies on the same bead. Beads were coated with anti-CD3 (OKT3) and anti-CD28 9.3 monoclonal antibody with each antibody added at 150 femtograms per bead. It is important to note that no cytokines were added to the culture medium so that cell growth was dependent on secretion of cytokines and lymphokines. Fresh medium was

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added at two to three days intervals with fresh medium to maintain cell concentrations between  $0.5-1.5 \times 10^6$  T cells/ml; antibody-coated beads and CHO cells were not cleared from culture, but were diluted progressively until restimulation. The cell cultures were monitored by electronic cell sizing using a ~~Coulter Counter model ZM~~ COULTER COUNTER® ZM and Channelyzer model 256 (Coulter, Hialeah, Fla.), and restimulated at approximately 7 to 10 day intervals (i.e. when the volume of the T cell blasts decreased to <400 fl) with additional beads and mitomycin C-treated CHO cells. Viable T cells were counted and the total number of cells that would be expected to accumulate displayed, taking into account discarded cells. - -

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